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The Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptors TRAIL-R1 and TRAIL-R2 Have Distinct Cross-linking Requirements for Initiation of Apoptosis and Are Non-redundant in JNK Activation*

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Overexpression of the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors, TRAIL-R1 and TRAIL-R2, induces apoptosis and activation of NF-κB in cultured cells. In this study, we have demonstrated differential signaling capacities by both receptors using either epitope-tagged soluble TRAIL (sTRAIL) or sTRAIL that was cross-linked with a monoclonal antibody. Interestingly, sTRAIL was sufficient for induction of apoptosis only in cell lines that were killed by agonistic TRAIL-R1- and TRAIL-R2-specific IgG preparations. Moreover, in these cell lines interleukin-6 secretion and NF-κB activation were induced by cross-linked or non-cross-linked anti-TRAIL, as well as by both receptor-specific IgGs. However, cross-linking of sTRAIL was required for induction of apoptosis in cell lines that only responded to the agonistic anti-TRAIL-R2-IgG. Interestingly, activation of c-Jun N-terminal kinase (JNK) was only observed in response to either cross-linked sTRAIL or anti-TRAIL-R2-IgG even in cell lines where both receptors were capable of signaling apoptosis and NF-κB activation. Taken together, our data suggest that TRAIL-R1 responds to either cross-linked or non-cross-linked sTRAIL which signals NF-κB activation and apoptosis, whereas TRAIL-R2 signals NF-κB activation, apoptosis, and JNK activation only in response to cross-linked TRAIL.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL),[1] also designated as APO-2 ligand, is a member of the tumor necrosis factor (TNF) family that is capable of inducing apoptosis in several cell lines (1, 2). TRAIL is widely expressed in normal cells and is highly homologous to FasL, another cytotoxic member of the TNF ligand family (1, 2). In addition to an involvement of TRAIL in natural killer cell-, dendritic cell-, and CD4+ T-cell-mediated cytotoxicity (3–6), TRAIL may also be involved in monocyte-mediated tumoricidal activity (7) and activation-induced T cell death during HIV infection (8, 9).

Currently five human TRAIL receptors belonging to the TNF receptor superfamily have been identified. Two of them, TRAIL-R1 (DR4, Ref. 10) and TRAIL-R2 (DR5, TRICK2, KILLER; see Refs. 11–18) contain a cytoplasmic death domain and transmit an apoptotic signal in response to TRAIL. Two other cellular TRAIL receptors, TRAIL-R3 (TRID, DcR1; see Refs. 11, 14, 17, 19), which is glycosylphosphatidylinositol (GPI)-linked and TRAIL-R4 (DcR2; see Refs. 20, 21), which contains a truncated death domain, bind TRAIL without activation of the apoptotic machinery and seem to antagonize the death domain-containing TRAIL receptors. In addition, osteoprotegerin, a regulator of osteoclastogenesis, is a soluble receptor for TRAIL (22). TRAIL-R1- and TRAIL-R2-mediated apoptosis occurs via activation of caspase-8 and subsequent activation of effector caspases. However, the link between the death domain-containing TRAIL receptors and activation of caspase-8 is rather undefined. Transient transfection of TRAIL-R1 leads to activation of the apoptotic machinery in Fas-associated death domain protein-deficient fibroblasts, suggesting that FADD, a death domain adapter molecule, is not required for TRAIL-R1-induced apoptosis (23, 24). On the other hand, overexpression of a dominant-negative FADD mutant was shown to block TRAIL-mediated apoptosis (12, 13, 15, 25). It has not yet been clarified whether FADD plays a specific role in TRAIL-R2-induced apoptosis or whether a closely related adapter protein is involved in TRAIL-R1- and TRAIL-R2-mediated apoptosis.

Because TRAIL is highly effective in killing cancer cell lines but has apparently no lethal effects on normal cells, TRAIL and its apoptotic receptors have attracted much attention as targets for anti-cancer therapy (26, 27). In this study, we show that TRAIL-R1 and TRAIL-R2 have different capabilities for stimulating the JNK pathway and differ also in their cross-linking requirements for activation by recombinant ligands. This is the first reported evidence of a difference between TRAIL-R1 and TRAIL-R2 signaling activities.

EXPERIMENTAL PROCEDURES

Materials—The anti-FLAG monoclonal antibody M2 was purchased from Sigma-Aldrich (Deisenhofen, Germany). Polyclonal sera specific for JNK, p65, p50, and cRel were purchased from Santa Cruz Biotechnology (Heidelberg, Germany) and protein A-Sepharose was from Amersham Pharmacia Biotech (Freiburg, Germany). The SuperFect transfection reagent was obtained from Qiagen (Hilden, Germany). TRAIL-R1-Fc and TRAIL-R2-Fc were from Alexis (Läufelfingen, Switzerland).

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[1] The abbreviations used are: TRAIL, TNF-related apoptosis-inducing ligand; JNK, c-Jun N-terminal kinase; TNF, tumor necrosis factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TRAIL-R, TRAIL receptor; FACS, fluorescence-activated cell sorter; CHX, cycloheximide; EMSA, electrophoretic mobility shift assay; Z-VAD-fmk, benzyloxycarbonyl-VAD-fluoromethylketone; mAb, monoclonal antibody; IL-6, interleukin-6; GST, glutathione *S*-transferase; ELISA, enyzme-linked immunosorbent assay.

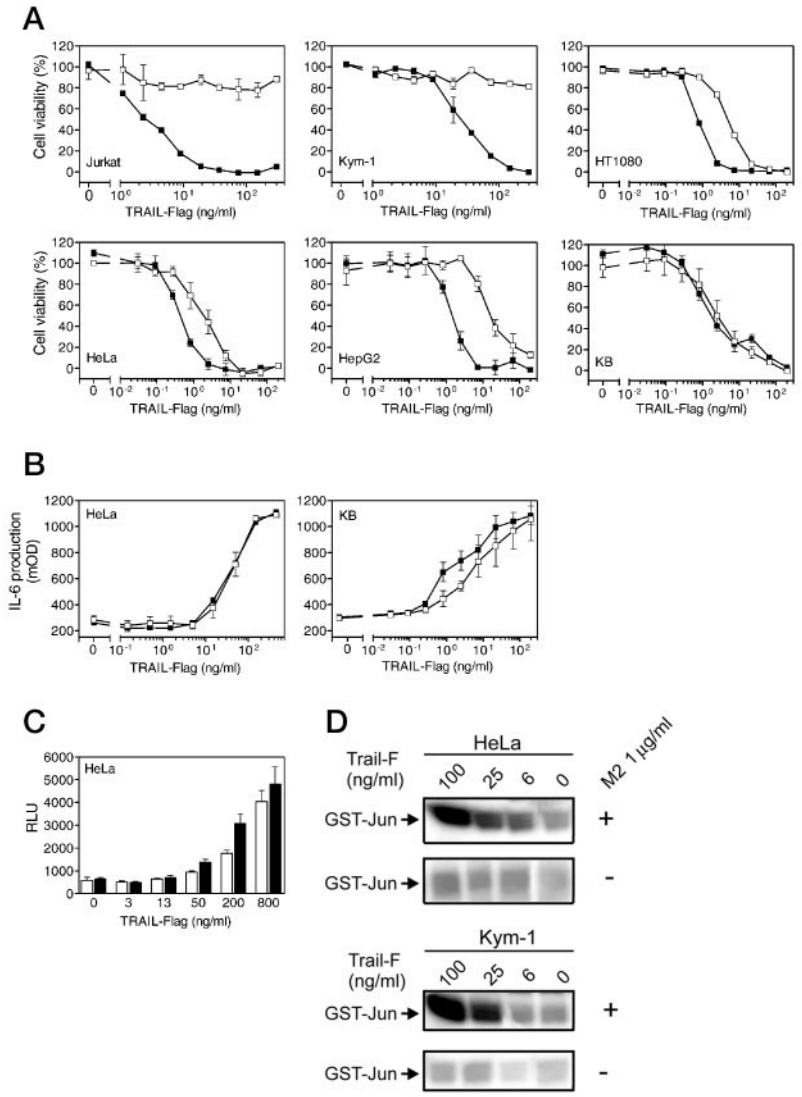

FIG. 1. **Cell type- and response-specific effects of cross-linked TRAIL-FLAG.** *A,* the indicated cell lines were analyzed for their sensitivity to FLAG-tagged sTRAIL in the presence (*filled squares*) or absence (*open squares*) of aggregating anti-FLAG antibody M2. Cell viability was determined using the MTT assay (Jurkat, Kym-1) or by staining adherent cells with crystal violet (HeLa, KB, HepG2, HT1080). The difference in absorbance between dead and living cells was in the range of 600–1000 mOD units for all cells. *B*, HeLa and KB cells were cultured overnight in 96-well plates. Cells were then incubated for 18 h with the indicated concentrations of cross-linked (*filled squares*) and non-cross-linked (*open squares*) sTRAIL-FLAG in the presence of 2.5 μg/ml CHX and 10 μM Z-VAD-fmk. Finally, IL-6 concentrations in the supernatants were determined using a commercially available ELISA kit. *C*, HeLa cells were cultured overnight in 96-well plates. The next day, cells were transfected with a 3× NF-κB-luciferase reporter plasmid and a SV40 promoter-driven β-galactosidase expression plasmid to normalize the transfection efficiency. After an additional day, cells were stimulated for 9 h with cross-linked (*filled bars*) and non-cross-linked TRAIL-FLAG (*open bars*) in the presence of CHX (2.5 μg/ml) and Z-VAD-fmk (10 μM). Finally cells were assayed for NF-κB activation. *D*, cell lysates were prepared from HeLa and Kym-1 cells that had been stimulated for 4 h with the indicated concentrations of cross-linked and non-cross-linked TRAIL-FLAG (*TRAIL-F*). JNK activity was measured by immunocomplex kinase assay with GST-c-Jun-(1–79) as substrate.

Cell Lines—HeLa, HepG2, and Jurkat cells were maintained in RPMI 1640 medium containing 5% (HeLa, HepG2) or 10% (Jurkat) heat-inactivated fetal calf serum. KB cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and HT1080 cells in Dulbecco's modified Eagle's medium-nutrient mix F12 containing 10% fetal calf serum. The Kym-1 cell line was maintained in Click RPMI 1640 medium supplemented with 10% fetal calf serum.

Generation of TRAIL-R1 and TRAIL-R2-specific IgG Preparations—Using a commercial antibody production service (Eurogentec, Seraing, Belgium), rabbits were immunized with TRAIL-R1-Fc and TRAIL-R2-Fc. For antibody purification, TRAIL-R1-Fc and TRAIL-R2-Fc were coupled to HiTrap *N*-hydroxysuccinimide (NHS)-Sepharose (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's protocol. Fc-specific antibodies were first depleted by repeated passages over human IgG1-agarose (Sigma, Deisenhofen, Germany). TRAIL-R1/R2-specific antibodies were further purified on TRAIL-R1/R2-Fc-Sepharose, eluted in 50 mM citrate/NaOH, pH 2.7, neutralized with Tris-HCl, pH 9, and dialyzed against phosphate-buffered saline. At concentrations below 500 ng/ml, we found no evidence for cross-reactivity of the anti-TRAIL receptor IgGs even upon secondary cross-linking with protein A. At higher concentrations (>1000 ng/ml) we observed a significant cross-reactivity of the anti-TRAIL-receptor IgGs that could be blocked by addition of TRAIL-R1-Fc to anti-TRAIL-R2 IgG and vice versa (data not shown).

Cytotoxic Assays—50,000 (Jurkat), 30,000 (HepG2, KB), 20,000 (HeLa, HT1080) or 15,000 Kym-1 cells were grown overnight in 100 μl of culture medium in 96-well plates. The cells were then treated for 16 h with FLAG-tagged TRAIL, TRAIL-M2-complex, anti-TRAIL-R1- and anti-TRAIL-R2-IgG. Cell death assays with HepG2, HeLa, KB, and HT1080 cells were performed in the presence of 2.5 μg/ml cycloheximide (CHX). TRAIL-M2-complex was generated by mixing the respective concentration of FLAG-tagged TRAIL with the anti-FLAG monoclonal antibody M2 to a final concentration of 1 μg/ml of antibody. After a 15-min incubation at room temperature, the TRAIL-M2-complex was transferred to the cells. TRAIL-R1 and TRAIL-R2 IgG were added in the presence of 1 μg/ml protein A (Sigma, Deisenhofen, Germany). Cell viability was determined using the MTT method (Jurkat, Kym-1) or crystal violet staining (HepG2, HeLa, KB, HT1080) as described previously (28, 29).

Immunocomplex JNK Assay—N-terminal c-Jun kinase assays were performed upon immunoprecipitation of JNK1 using a rabbit antiserum (Santa Cruz Biotechnology, Heidelberg, Germany). GST-c-Jun was used as substrate in an *in vitro* kinase assay as described previously (30).

Transient Reporter Gene Assays—For transient reporter gene assays, 20,000 HeLa cells were seeded in 96-well tissue culture plates, and the following day the cells were transfected with a 3× NF-κB-luciferase reporter plasmid (15% transfected DNA), a SV40 promoter-driven β-galactosidase expression plasmid (5% transfected DNA) to normalize the transfection efficiency, and empty vector (80% transfected DNA). Transfections were performed with SuperFect reagent according to the manufacturer's recommendations (Qiagen, Hilden, Germany). After a 1-day recovery, cells were treated with TRAIL, TRAIL-M2-complex, CHX, and Z-VAD-fmk as indicated, harvested in phosphate-buffered saline, and then luciferase and β-galactosidase activities were determined using the Galacto-Light Plus reporter gene assay kit (Perkin Elmer, Nieuwerkerk, The Netherlands) and a Lucy2 96-well luminometer (Anthos, Krefeld, Germany).

Determination of Interleukin-6 Production—Cells (1.5×10^4 per well) were seeded in triplicates in 96-well tissue culture plates in 100 μl of Click RPMI 1640 and cultured overnight. The following day the cells were treated with the reagents of interest as indicated for an additional 12–24 h. Then the supernatants were removed, cleared by centrifuga-

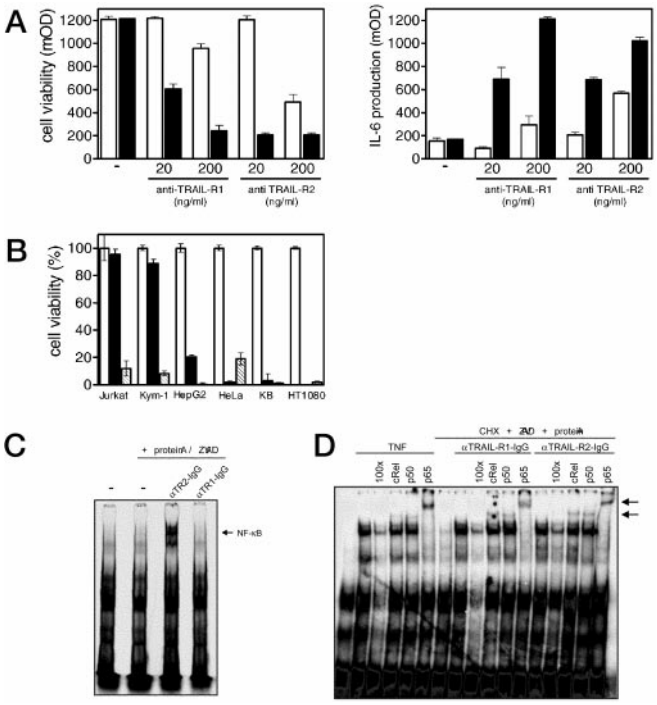

FIG. 2. *A*, protein A cross-linking enhanced the agonistic capacity of anti-TRAIL-R1 and -R2 IgG. HepG2 cells were incubated overnight with the indicated concentration of anti-TRAIL-R1 IgG and anti-TRAIL-R2 IgG, respectively, with (*filled bars*) or without (*open bars*) previous aggregation with protein A in the presence of 2.5 μg/ml CHX. Cell viability was measured by crystal violet staining. In addition, HeLa cells were treated in the same way with the anti-TRAIL-receptor IgGs in the presence of 2.5 μg/ml CHX and 10 μM Z-VAD-fmk, and supernatants were analyzed for production of IL-6. *B*, cytotoxic effects of anti-TRAIL-R1 IgG and anti-TRAIL-R2 IgG. Various cell lines were incubated overnight with protein A cross-linked anti-TRAIL-R1 IgG (*filled bars*) or anti-TRAIL-R2 IgG (*hatched bars*) or were left untreated (*open bars*). The next day, cell viability was determined by the MTT assay (Jurkat, Kym-1) or by staining with crystal violet (HeLa, KB, HepG2, HT1080). To allow induction of apoptosis, HepG2, HeLa, KB, or HT1080 cells were treated in the presence of 2.5 μg/ml CHX. *C*, Kym-1 cells were treated with anti-TRAIL-R1 or anti-TRAIL-R2 IgG (*αTR1-IgG* or *αTR2-IgG*, respectively; 200 ng/ml) and protein A (1 μg/ml) with Z-VAD-fmk (*ZVAD*, 20 μM). After 3 h, cells were harvested and analyzed for NF-κB activation by EMSA. *D*, HeLa cells were treated as indicated with, anti-TRAIL-R1 and anti-TRAIL-R2 IgG (200 ng/ml), protein A (1 μg/ml), CHX (2.5 μg/ml), and Z-VAD-fmk (20 μM) for 3 h. As a control, cells were also treated with 10 ng/ml TNF for 30 min. Supershift analyses were performed as described under "Experimental Procedures." Supershifted complexes of the NF-κB oligonucleotide and p65, p50, or cRel, respectively, are indicated by *arrows*.

tion (15,000 rpm, 10 min, 4 °C) and interleukin-6 concentrations were determined using a commercially available ELISA kit (PharMingen, Hamburg, Germany).

FACS Staining—Cells were stained for TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 expression in 100 μl of FACS buffer (phosphate-buffered saline, 5% fetal calf serum, 0.1% NaN_3) with 5 μg/ml anti-TRAIL-R1 mAb M271 (IgG2a), anti-TRAIL-R2 mAb M413 (IgG1), anti-TRAIL-R3 mAb M430 (IgG1) and anti-TRAIL-R4 mAb M445 (IgG1), respectively, or the respective control IgG, followed by fluorescein isothiocyanate-labeled anti-mouse antibody (5 μg/ml). FACS analyses were performed with a FACStar plus instrument (Becton Dickinson, San Jose, CA).

EMSA Analysis of NF-κB Activation—HeLa and Kym-1 cells (10^6) were seeded in 60-mm cell culture dishes and cultivated overnight to allow adherence. The next day the cells were stimulated for 3 h with the indicated combinations of anti-TRAIL-R1 and anti-TRAIL-R2 IgG, protein A (1 μg/ml), Z-VAD-fmk (20 μM) and CHX (2.5 μg/ml). Nuclear extracts were prepared as described previously (31), and EMSA analyses were performed using a standard procedure with a high pressure liquid chromatography-purified NF-κB-specific oligonucleotide (5′-ATC AGG GAC TTT CCG CTG GGG ACT TTC CG-3′), end-labeled with [^{32}P]ATP. Finally, samples were separated by native polyacrylamide gel

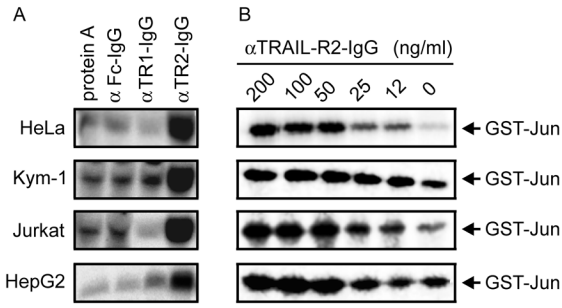

FIG. 3. **TRAIL-R2 but not TRAIL-R1 signals JNK activation.** *A*, cell lysates were prepared from Kym-1, Jurkat, HeLa, and HepG2 cells that had been stimulated for 4 h with protein A cross-linked (1 μg/μl) anti-TRAIL-R1 IgG and anti-TRAIL-R2 IgG, each at 200 ng/ml in the absence of CHX. For control purposes lysates from cells treated with protein A and non-relevant IgG were also analyzed. JNK activity was measured by immunocomplex kinase assay with GST-c-Jun-(1–79) as a substrate. *B*, dose dependence of anti-TRAIL-R2 IgG-induced JNK activation. Kym-1, Jurkat, HeLa, and HepG2 cells were stimulated with the indicated concentrations of protein A cross-linked anti-TRAIL-R2 IgG for 4 h, and lysates were analyzed for JNK activity by immunocomplex kinase assays.

electrophoresis in low ionic strength buffer. For supershift analyses, 10 μl of the nuclear extracts (1 μg/μl protein) were incubated on ice for 1 h with 1 μg of polyclonal antibodies specific for p65, p50, and cRel (Santa Cruz Biotechnology, Heidelberg, Germany). Then the formed complexes were incubated with 2 μl of 5× binding buffer (500 mM KCl, 50 mM Tris-HCl, pH 7.4, 25 mM $MgCl_2$, 50% glycerol, 5 mM dithiothreitol) and 2 μl of poly(dI-dC) (2 mg/ml) in a final volume of 20 μl. NF-κB DNA-binding activity was again analyzed by native polyacrylamide gel electrophoresis and phosphorimaging (Storm 860; Amersham Pharmacia Biotech, Freiburg, Germany).

RESULTS AND DISCUSSION

Most ligands of the TNF family are either membrane-bound or proteolytically processed into soluble proteins. Evidence suggests that artificial cross-linking of soluble ligands mimics the distinct biological activities of the corresponding membrane-bound ligands. For example, we have recently shown that the cytotoxic activity of FLAG-tagged human Fas ligand (sFasL), was increased by >1000-fold in response to cross-linking with the anti-FLAG monoclonal antibody M2. Notably, this increased activity was comparable with the cytotoxic potency of membrane-bound FasL (32). Further, activation of TNF-R2-dependent signaling pathways by soluble FLAG-tagged TNF was strongly increased by multimerization of this ligand by the anti-FLAG monoclonal antibody M2. In accordance with that, we have previously shown that membrane-bound, but not soluble TNF, is the prime activating ligand for TNF-R2 (33, 34), suggesting that cross-linked and membrane-bound ligands have analogous effects on this receptor. Using various cell lines we have therefore tested whether a recombinant soluble FLAG-tagged form of TRAIL (sTRAIL) required cross-linking for its activity.

We found that several cell lines, *e.g.* Jurkat and Kym-1, designated in the following as group I cells, were killed by physiological amounts (<200 ng/ml) of sTRAIL only in the presence of secondary cross-linking by the anti-FLAG monoclonal antibody M2 (Fig. 1*A*). However, we also identified a second set of cell lines designated in the following as group II cells that were efficiently killed by non-cross-linked sTRAIL (HeLa, HepG2, HT1080, and KB; Fig. 1*A*). The group II cell lines Hela and KB were also tested with respect to the cross-linking requirements of sTRAIL for non-apoptotic signaling. As shown in Fig. 1, cross-linked and non-cross-linked sTRAIL both have a comparable capacity to induce IL-6 production (Fig. 1*B*) and elicited comparable NF-κB activation in a reporter gene assay (Fig. 1*C*). The magnitude of NF-κB activation and IL-6

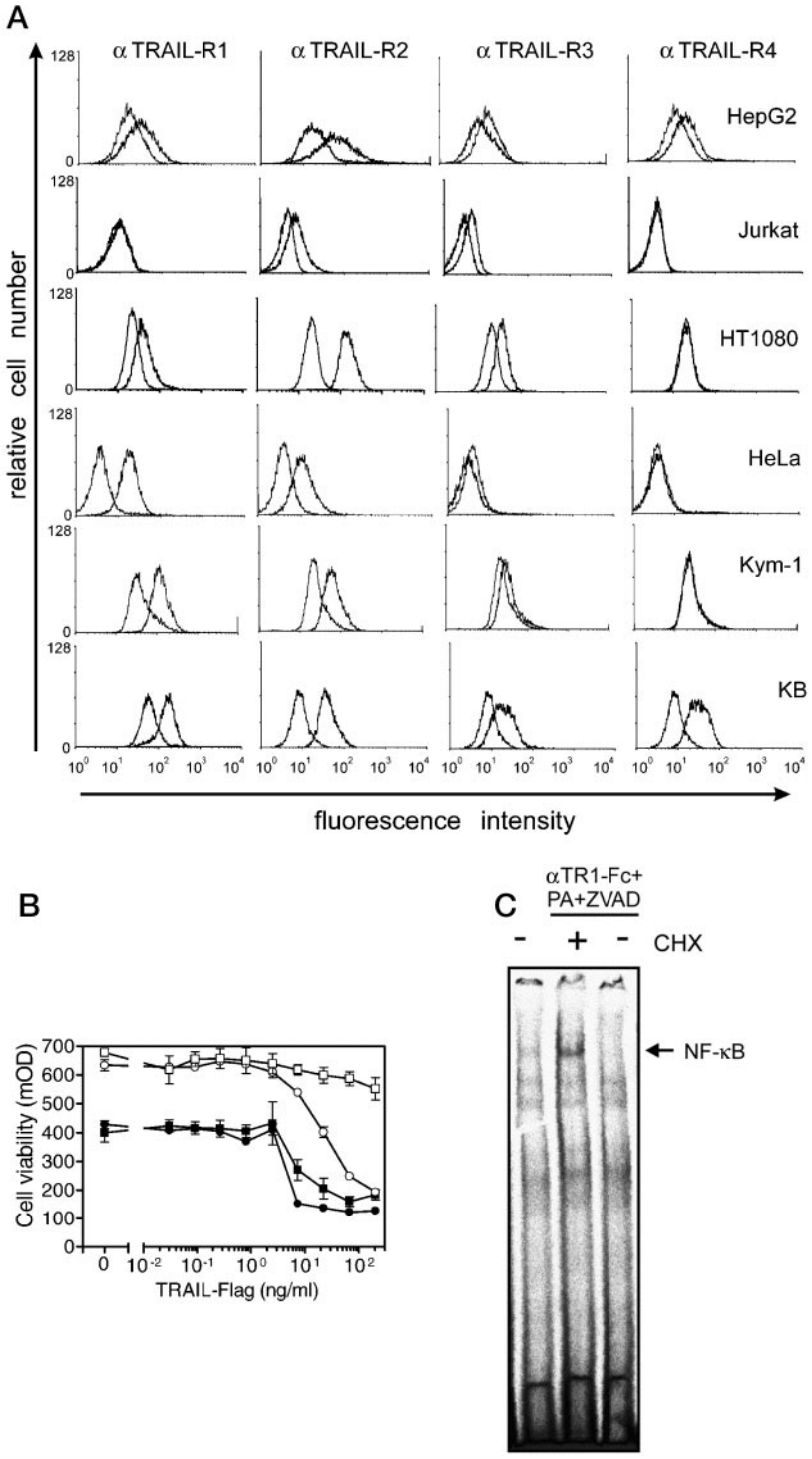

FIG. 4. *A*, analysis of TRAIL receptor expression on various cell lines by FACS. *B*, effect of CHX on the killing of Kym-1 cells by cross-linked and non-cross-linked sTRAIL. Kym-1 cells were cultured overnight in 96-well plates. Cells were then incubated for additional 18 h with the indicated concentrations of cross-linked (*circles*) and non-cross-linked (*squares*) sTRAIL in the presence (*filled symbol*) or absence (*open symbol*) of 2.5 μg/ml CHX. Cell viability was measured by the MTT assay. *C*, Kym-1 cells were treated with anti-TRAIL-R1 IgG (200 ng/ml), protein A (1 μg/ml), and Z-VAD-fmk (*ZVAD*, 20 μM) in the presence or absence of 2.5 μg/ml CHX. After 3 h, cells were harvested and analyzed for NF-κB activation with EMSA.

production induced by cross-linked and non-cross-linked sTRAIL was similar to that obtained by TNF stimulation (data not shown). In all group II cells investigated, TRAIL-induced apoptosis and activation of NF-κB were dependent on the presence of CHX. Activation of the NF-κB pathway is inhibited by caspase-dependent mechanisms during apoptosis (35–38). Thus, TRAIL-induced activation of NF-κB was therefore only observed in group II cells when in addition to CHX a caspase inhibitor (Z-VAD-fmk) was present (data not shown). However, in group I cells, NF-κB activation was found in the absence of CHX, provided that apoptosis was again inhibited by Z-VAD-fmk (data not shown). Notably, when we analyzed TRAIL-mediated JNK activation in group I (Kym-1) and II cells (HeLa), we found in both cell lines a requirement for cross-linked sTRAIL (Fig. 1*D*). As already outlined above, in group II cell lines, sTRAIL activated NF-κB only in the presence of CHX/Z-VAD-fmk and induced cell death only if CHX was present. However, activation of the JNK pathway by cross-linked TRAIL occurred in the absence of CHX and was therefore not linked to cell death.

To analyze whether the requirement for cross-linked sTRAIL correlated with a differential utilization of TRAIL-R1 and TRAIL-R2, we reexamined the cells described above using purified IgG fractions of agonistic TRAIL-R1- and TRAIL-R2-specific antisera. The agonistic activity of anti-TRAIL-R1 IgG and anti-TRAIL-R2 IgG, respectively, was significantly in-

creased upon aggregation with protein A, with respect to death induction and up-regulation of IL-6 production (Fig. 2*A*). At the concentrations used in this study (<200 ng/ml) the IgG fractions were not cross-reactive. Using the agonist anti-TRAIL-receptor IgGs, we found that group I cells were exclusively killed by anti-TRAIL-R2 IgG, whereas group II cells were sensitive for stimulation with both anti-TRAIL-R1 and anti-TRAIL-R2 IgG (Fig. 2*B*). Moreover, in the group II cell line HeLa, both IgG preparations induced NF-κB activation whereas in the group I cell line Kym-1 only anti-TRAIL-R2 IgG but not anti-TRAIL-R1 IgG was able to activate NF-κB (Fig. 2, *C* and *D*). Again in HeLa cells treatment with CHX and Z-VAD-fmk was necessary to elicit the NF-κB response, whereas in Kym-1 cells NF-κB activation only required inhibition of the apoptotic pathway. Supershift analyses in HeLa cells revealed that TRAIL-R1 and TRAIL-R2 engaged the NF-κB family members p65, p50, and cRel in a comparable manner to TNF-R1 (Fig. 2*D*). In group I as well as in group II cell lines the first signs of NF-κB DNA-binding activity were detectable 1 to 2 h upon TRAIL receptor stimulation whereas TNF induced NF-κB DNA-binding activity within 15–30 min. NF-κB DNA-binding activity induced by both cytokines sustained for several hours in both type of cells (data not shown). Importantly, the JNK pathway was triggered in group I and II cell lines by stimulation of TRAIL-R2 but not by stimulation of TRAIL-R1 (Fig. 3). To our knowledge, this is the first reported difference in the signaling capacities of the two death domain-containing TRAIL receptors.

Based on these results, it is evident that group II cells must co-express both death domain-containing TRAIL receptors, whereas group I cells either express no TRAIL-R1 or this molecule was silenced in some way under the conditions used in our study. As shown in Fig. 4*A*, all cells investigated with the exception of Jurkat cells were positive for TRAIL-R1 and TRAIL-R2 expression in FACS analysis. In addition, with the exception of Jurkat and HeLa cells, all investigated cell lines express at least one of the TRAIL decoy receptors (TRAIL-R3, TRAIL-R4). Nevertheless, in all cases the expression of the decoy receptors was rather low compared with TRAIL-R1 and TRAIL-R2 expression, which is consistent with the TRAIL-sensitivity of these cell lines. In light of the expression data it became clear that the group I cell line Jurkat did not respond to anti-TRAIL-R1 IgG (or non-cross-linked sTRAIL) as TRAIL-R1 is not significantly expressed on this cell line. However, in the case of the TRAIL-R1-expressing Kym-1 cell line, it is obvious that TRAIL-R1 signaling has to be negatively regulated (by an unknown mechanism). As in group II cells, because low concentrations of the metabolic inhibitor CHX were necessary to allow TRAIL-R1-mediated NF-κB activation and induction of cell death, we tested the signaling capacity of TRAIL-R1 in this group I cell line also in the presence of CHX. In fact, Kym-1 cells became sensitive to anti-TRAIL-R1 IgG (data not shown) and non-cross-linked sTRAIL (Fig. 4*B*) in the presence of CHX. Moreover, whereas in the absence of CHX only stimulation of TRAIL-R2 activated the NF-κB pathway (see Fig. 2*C*), stimulation of TRAIL-R1 also induced NF-κB activation provided that CHX and Z-VAD-fmk were added (see Fig. 4*C*). Because of the high cytotoxicity of CHX, putative effects of this compound on TRAIL receptor-induced apoptosis, cells could not be examined in Jurkat cells (data not shown). Our observations may suggest the existence of two CHX-sensitive factors or pathways. The first one is active in group II cells to prevent TNF-, FasL-, and TRAIL-mediated cell death. The second ligand may specifically block TRAIL-R1 pathways in group I cells. It is noteworthy that TRAIL-R2-mediated JNK activation occurred in group I and group II cells in the absence of CHX, clearly demonstrating the ability of TRAIL-R2 to transmit specific signals in the absence of cell death. It is possible that TRAIL-R2 is also important for non-apoptotic signal transduction. This may involve the activation of c-Jun and other JNK- or NF-κB related downstream responses, which regulate proliferation and differentiation in normal cells. The apoptotic function of TRAIL-R2, which is cryptic in normal cells, may only be dominantly revealed in transformed cells. Although JNK was only activated *via* endogenous TRAIL-R2 but not endogenous TRAIL-R1, we also noted that transient overexpression of both TRAIL-R1 and TRAIL-R2 activated JNK in a ligand-independent fashion (data not shown). We can therefore not completely exclude the possibility that endogenous TRAIL-R1 might activate JNK in some circumstances.

In conclusion, our data suggest that TRAIL-R1 responds to cross-linked and non-cross-linked TRAIL to signal NF-κB activation and apoptosis, whereas TRAIL-R2 signals NF-κB activation, apoptosis, and JNK activation in response to cross-linked TRAIL only. We hypothesize that the requirement of cross-linked sTRAIL reflects the requirement of TRAIL-R2 for membrane-bound TRAIL.

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